INTRODUCTION TO BIOCATALYSIS USING ENZYMES AND MICRO-ORGANISMS

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An historical introduction to biocatalysis using enzymes and micro-organisms

1.1 Background

Each year that passes sees increasing interest in the application of enzymes and micro-organisms as catalysts in organic chemistry. Well-received national reports on the potential growth of biotechnology, not to speak of numerous reviews of the topic of biotransformation itself, have described the advantages which biological catalysis can bring to complex organic syntheses. At this time it is indeed an exciting prospect for anyone who has a rôle to play in the development of this new technology. But occasionally a prospective participant, as well as a reviewer, ought to stand back and ask, Why has this explosion of interest happened now?

An answer to such a question often lies in the historical context from which technologies and their underlying science develop. This is a topic worthy of greater attention than it usually receives from practising scientists. A resort to scientific papers more than one or two decades old is frequently viewed as perverse, while to consult the scientific literature of the past century in search of a pertinent contemporary lesson is simply eccentric. Yet this old literature can be worth reading for the light it can shed on current research, quite apart from which, it is, more often than not, a pleasure to read.

The biochemistry of the late nineteenth century has an interesting connection with the manufacture of vitamin C. The basis of this process relied on the development of the concepts of fermentation and catalysis, but it emerged directly from a microbial oxidation described in a short paper written by Adrian Brown (1886a). The background to his work, and its industrial setting, can provide the focus for a broader introduction to the use of enzymatic catalysis in modern organic chemistry. It will also suggest an answer to the question posed earlier.

For anyone interested in reading more extensively, a wide selection of the relevant nineteenth-century literature is not usually available, except in the older universities. However, the books by Boyde (1980) and by Teich and Needham (1992) contain many of the key papers translated into English, connected with a commentary.

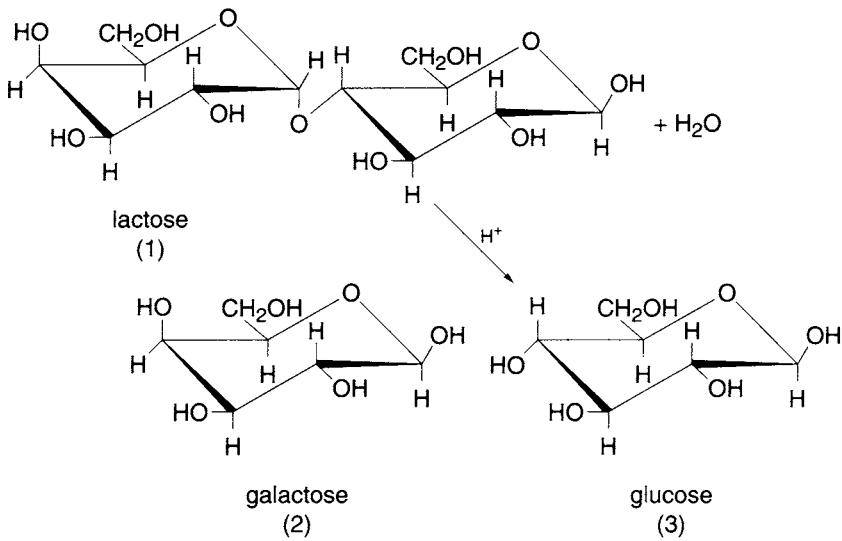
1.2 Kirchhoff and starch hydrolysis

In the early nineteenth century, developments in biochemistry were closely associated with those in organic chemistry. Moreover, the science of these subjects was not distinct from the technology of their application. The chemical and biochemical reactions in which starch and sugar participated were important topics for scientific research and technological development. The trail from that period to the current methods for manufacture of ascorbic acid (vitamin C) may as well begin with a note about the ink with which a chapter such as this would once have been written. It would at one time have contained gum arabic, which was, and still is, an expensive natural carbohydrate. As an alternative, Bouillon-Lagrange (1811) described a method for preparing inks using a modified starch. He heated dry starch powder gently until it began to char. This material formed a suitable gum in water, which he noted to have a sweet taste. He further treated the gum with acids, but they were too strong to have enhanced the sweet flavour, since sulphuric acid caused further charring and gave off a smell of acetic acid; in contrast, nitric acid formed oxalic acid.

The preparation of that gum is worth recording, because in the following year Kirchhoff showed that if starch was boiled in dilute sulphuric acid, the suspension became sugary (Teich and Needham, 1992). That sugar (Vogel, 1812) was soon shown to be fermentable; moreover, the same acid treatment converted the sugar in milk [lactose (1)] into a fermentable form through its hydrolysis into galactose (2) and glucose (3) (Scheme 1.1) (Vogel, 1817).

It is easy to write in terms of definite chemicals entities after an interval of nearly two centuries. It is wrong, even demeaning of their achievements, to imagine those chemists as having anything more than their contemporary understanding of the experiments. Nevertheless, the importance of this discovery, that the acid treatment of starch would convert it to sugar, was clearly recognized. Not only was the conversion

interesting in its own right, perhaps in shedding light on the chemistry and physiology of several processes in plants; but it also gives society a product, which



Scheme 1.1. Hydrolysis of lactose.

in many circumstances could replace the cane sugar, which is already expensive, and whose price rises incessantly.
(De Saussure, 1814, p. 499)

At the time, organic chemists found these materials very difficult to study. The simple elemental ratios which were the rule in the compounds of inorganic origin were not found in organic chemistry. Nevertheless, it was clear that in decomposing the starch into sugar, the sulphuric acid did not enter into the product, nor was it consumed in the process. It was correctly inferred from the elemental analysis that only the elements of water were entering the starch during the conversion (Table 1.1).

The influence of the acid in this operation appears to be limited to increasing the fluidity of the aqueous solution of the starch, so helping the latter to combine with water.
(De Saussure, 1814, p. 501)

The fractional ratios between the elements in the materials isolated from living matter convinced some that the chemical processes associated with their synthesis were somehow different from those associated with inorganic matter and incorporated some vital factor:

All simple bodies in nature are subject to the action of two powers, of which one, that of attraction, tends to unite the molecules of bodies one with another, while the other, produced by caloric, forces them apart.... A certain number of these

Table 1.1. *Elemental analysis of starch and of the sugar recovered after treatment with sulphuric acid*

Sample	C	O	H	N
Starch				
De Saussure observed	45.39	48.31	5.90	0.40
$(C_6H_{10}O_5)_n$ theory[a]	44.5	49.3	6.2	—
Released sugar				
De Saussure observed	37.29	55.87	6.84	—
$C_6H_{12}O_6$ theory[a]	40.0	53.3	6.7	—
$C_6H_{12}O_6 \cdot \frac{1}{2}H_2O$ theory[a]	38.1	55.0	6.9	

[a]Calculated values based on the given elemental compositions.
Source: De Saussure (1814).

simple bodies in nature are subject to a third force, to that caused by the vital factor (*le principe vital*), which changes, modifies and surpasses the two others, and whose limits are not yet understood.
(Beral, 1815, pp. 358–9)

In the discussion which followed that paper (p. 361), one of the journal's editors, J.-J. Virey, pointed out that there were some products of inorganic chemistry that also had poorly defined compositions, notably the oils formed when cast iron was treated with acid, or when olefinic gases were burnt. He also pointed out that some chemical processes (e.g. Kirchhoff's conversion of starch into sugar with acid) resembled the effects of germination and that respiration could release simple materials such as carbonic acid and water. Thus the distinction between organic and inorganic chemistry was not as clearly defined as Beral had portrayed it, and it warranted further study.

The chemical literature throughout the nineteenth century uses the term "body" in a sense which is similar to our contemporary use of the term "compound". (More generally, it is not prudent to assume that our modern definitions of chemical terms are the same as those once current.) Virey's remarks clearly illustrate one other feature of the chemistry of the early nineteenth century: that its experiments were much affected by impure reagents, even though the inferences drawn from them ring true in the late twentieth century.

1.3 Payen, Persoz and diastase

This early history of the hydrolysis of starch is the chemical background against which the technical study of catalysis and enzymology began.

Within a few years the conversions of starch and lactose were repeated with biological agents. Kirchhoff (1816) described the saccharification of starch in grain, and Vogel (1817) showed that an infusion of oats would produce a fermentable sugar from milk. Döbreiner (1815) used a wet yeast paste to convert sugar into "une liquer vineuse" which was no longer sweet.

The brewing industry must have played some part in determining the content of these experiments. It was already a large industry; Samuel Whitbread brewed 30,000 m^3 of beer in 1796. Moreover, the manufacture of ethanol was developing as a separate industry alongside brewing. In 1830 Aeneas Coffey, in Dublin, developed a distillation process with a multiplate countercurrent condenser (Figure 1.1) capable of recovering ethanol as an azeotrope with water from a fermented beer mash (Packowski, 1978). Biotechnology may have an ancient history, but this process, which incorporates the first large-scale use of a biochemical step in the manufacture of an organic reagent, can properly lay claim to be the first biotechnological process of modern chemistry.

The successful development of this technology is remarkable in the context of the very limited understanding of the process itself. The descriptions of nineteenth-century brewing practice suggest that it was anything but reliable; at Burton-on-Trent in Britain, little beer was brewed in the summer months (Brown, 1916) because the chance of spoilage was so high. The unreliable nature of the biocatalytic process was a direct consequence of this ignorance.

The first advance in understanding the process occurred in 1833 when Payen and Persoz extracted a mixture of amylases from malted barley. This soluble material ("soluble ferment") was able to separate and saccharify the starch away from the husk of the grain. They called the activity "diastase" (from the Greek word meaning "to separate"). The work was significant because it demonstrated that the "soluble ferment" could be dissolved out of the organised structures of the malted barley or the yeast, the so-called organized ferments. The saccharification of the starch which Kirchhoff had described could then be studied separately from the fermentation itself. The term "diastase" was eventually to be used as a description of any soluble enzyme, although it later reverted to an alternative description of an amylase.

Their research led to technological improvements in brewing practice, in which those authors participated:

> It will seem less remarkable that we have advanced only such a little distance along this new road if it is considered that we have been caught up in a millrace of

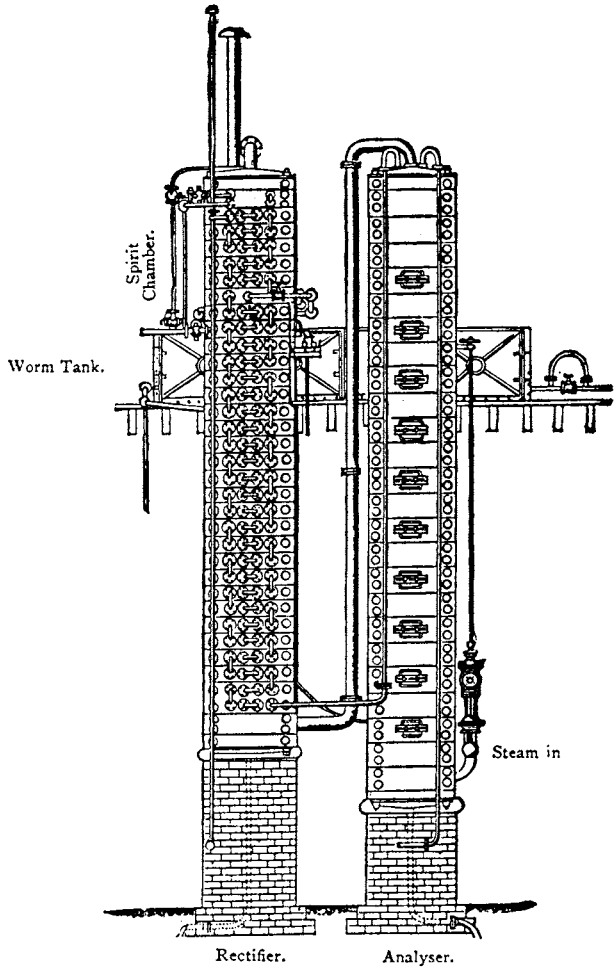


Figure 1.1. Coffey's distilling apparatus. The still has two columns, the analyser and the rectifier. Both are multiplate distillation units. Steam enters at the base of the analyser (Steam in), through which it rises. From the top it is piped to the lowest compartment of the rectifier, through which it again rises. The alcoholic mash enters through the top of the rectifier, through which it falls inside a long zigzag pipe which acts as a heat exchanger. From the bottom of the rectifier it is piped to the top of the analyser, through which it falls freely, mixing with the rising steam. It eventually leaves from the base of the analyser. The effect of this countercurrent process is to strip alcohol from the mash falling through the analyser against the flow of the steam. The multiplate arrangement ensures efficient removal of the alcohol from the mash. The alcohol-laden steam leaves the top of the analyser and enters the base of the rectifier, through which it rises against the flow in the zigzag cooling pipe containing the fresh mash. The multiple arrangement inside the rectifier ensures many stages of boiling and condensation so that the vapour at the top of the column is an azeotrope of water and ethanol. The upper section of the rectifier is the spirit

newborn applications, and that we have not thought it right to refuse our collaboration to the manufacturers who have requested it from every direction.
(*Payen and Persoz, 1833, p. 92*; see Boyde, 1980)

This was not surprising. Within two years there was an extensive paper comparing the effects of diastase and acid on a variety of starches, leading to the preparation of crystalline sugar (Guérin-Varry, 1835).

1.4 Berzelius and catalysis

The importance of the work by Payen and Persoz was clear to Berzelius. When he thoroughly revised his *Textbook of Chemistry* for the fourth German edition (Berzelius, 1838; the previous edition is dated 1831), he included the famous section on catalysis which he originally put forward in 1828 (Jorpes, 1966). Chemists were clearly puzzled by the organic processes in the living tissues. Berzelius compared them to a group of decomposition phenomena (Table 1.2) which could not be explained by the process of "double decomposition" in which two compounds reacted together. He suggested that some substances were characterized by

a new power to produce chemical activity belonging to both inorganic and organic nature [which] using a derivation well-known in chemistry [he called] the catalytic power of the substances, and decomposition by means of this power catalysis, just as we use the word analysis to denote the separation of the component parts of bodies by means of ordinary chemical forces.
(Berzelius, 1838; see Jorpes, 1966)

The implication that catalysis was a degradative power is intriguing, just as is the old use of the term "analysis," instead of the modern word "synthesis". It is also interesting to see what now we would regard as chemical and biochemical examples of catalysis freely taken together. The action of fibrin in degrading hydrogen peroxide (Table 1.2) presumably was due to a small amount of catalase with which it was contaminated.

Elsewhere (Jorpes, 1966), Berzelius wrote of diastase that:

One can hardly assume that this catalytic process is the only one in the vegetable kingdom. On the contrary, it gives reason to believe that within living plants and

◀

chamber, in which the constantly boiling mixture of the azeotrope is refluxed. The azeotrope leaves the rectifier from the bottom of this refluxing section and runs to the worm tank. The feints (a dilute solution of ethanol in water) which collect at the base of the rectifier are piped to the top of the analyser, there to be mixed with the mash. (From Nettleton, 1893; reproduced with the kind permission of the Institute of Brewing.)

Table 1.2. *Catalytic processes listed by Berzelius in 1838*

Catalytic process	Source of research
Acidic hydrolysis of starch to sugar	Kirchhoff
Decomposition of hydrogen peroxide in acid and alkali	Thenard
Decomposition of hydrogen peroxide by platinum and manganese dioxide	
Decomposition of hydrogen peroxide by fibrin	
Combustion in air of alcoholic and ethereal vapours on platinum	H. Davy
Effects of finely divided platinum	E. Davy
Ignition of hydrogen in air on platinum sponge	Döbreiner
Conversion of sugar into carbonic acid during yeast fermentation	

animals thousands of catalytic processes are going on between the tissues and the fluids, producing a multitude of chemical compounds, the creation of which out of the common raw material, the sap of plants or blood, has up to now been unexplained, and which may possibly be found in the future to depend on the catalytic power of the living tissues.
(Berzelius, 1838; see Jorpes, 1966)

1.5 Leibig and Hofmann

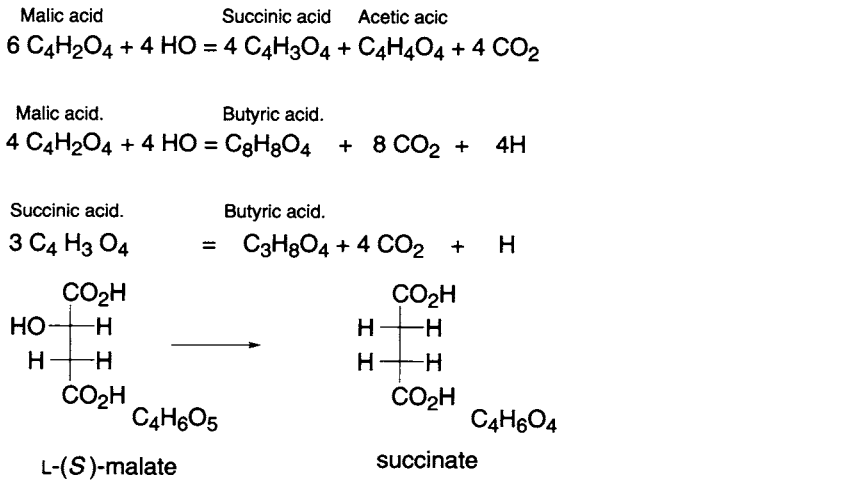
Berzelius had a poor view of British chemists (Jorpes, 1966). They nevertheless understood the importance of what he had written. When 10 years later Playfair (1848) reviewed the topic, he also took the decomposition of sugar by yeast as one of the examples with which to illustrate his views on the mechanism of catalysis. Perhaps this interest was partly the result of an influx of German chemists. In 1845, at Leibig's recommendation, Hofmann was appointed the director of the new Royal College of Chemistry in London (Travis, 1992; Leaback, 1992). That same year, another of Leibig's students, Henry Böttinger, left Germany, eventually to join the brewers Allsopp & Sons at Burton-on-Trent, where he was quickly appointed the head brewer.

Böttinger became friendly with Hofmann, and the students at the Royal College of Chemistry benefitted from the quantities of beer which he sent for analysis. When Payen suggested that the British brewers added strychnine to increase the bitterness of their beer, Böttinger turned to Hofmann for help. The report was written jointly with Thomas Graham, who was the professor of chemistry at University College London (Hofmann and Graham, 1852). It is, in retrospect, mildly amusing, because of the absurdity

of the claim, which is in contrast to the magisterial tone of its dismissal, but at the time it was no joke. The brewing of beer was a major industry throughout Europe, and the report was widely circulated (Armstrong, 1921, p. 244). Payen maintained that he had been misquoted by a journalist in France, a view which Hofmann and Graham accepted. Obviously the inaccurate reporting of scientific matters in the press is an old problem!

Neither Böttinger nor Hofmann was likely to understand the fermentation process while their outlook was limited by the all-pervasive views of their teacher, Leibig (Brown, 1916; Armstrong, 1921). He believed that it represented an inanimate interaction between the motions of the bodies of the yeast and the organic reagents. In that respect, Leibig's views seem not to be very different from those of Beral (1815) stripped of their reference to caloric.

Hofmann edited the English translation of Leibig's *Annual Report of Progress in Chemistry*. The report for 1847–8 contains details of Pasteur's physical separation of the crystals of the two isomers of tartaric acid, while that for 1849 reports Leibig's experiments on the fermentative conversion of malate into succinate. In an experiment lasting about three months, Dessaignes (1849) noted that crystals of calcium malate, held under water, were slowly converted into succinate. Leibig (Leibig and Kopp, 1849) showed that beer yeast, putrid fibrin and rotten cheese would also catalyse this change. As much as 15 or 16 ounces (about 0.43 kg) of succinic acid were obtained from 3 pounds (about 1.3 kg) of crude malate of lime

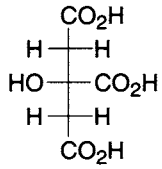


Scheme 1.2. The upper section of the scheme is taken directly from the original publication.

(calcium malate). Under other conditions, substantial amounts of acetic acid and butyric acid were also formed (Scheme 1.2). There is little reason to doubt these results, despite the problems with the elemental analysis of the products. (It is also noteworthy that the modern organic chemist would be more interested in the introduction of the chiral center than in its removal.)

Such transformations appeared regularly in the literature of the time, often as brief reports. Phipson (1862) compared the chemical and biological oxidations of citric acid. Permanganate was the chemical oxidant which produced oxalic acid; uncooked putrid beef and boiled beef were the biological agents which yielded butyric acid (Scheme 1.3). It is not surprising that the analysis of these transformations (Scheme 3) should differ from a modern interpretation.

Citric acid ($C_6H_8O_6$)



Oxalic acid ($C_2H_2O_4$)

$C_{12}H_8O_{14} + O_{12} = 3C_4H_2O_8 + 2HO.$
Citric Acid Oxalic Acid

$C_{12}H_8O_{14} = C_8H_8O_4 + 4CO_2 + O_2$
Citric Acid Butyric Acid

$CH_3CH_2CH_2CO_2H$
Butyric acid ($C_4H_8O_2$)

Scheme 1.3. The equations (only) are taken from the original publication.

The state of mid-nineteenth-century chemistry and its impact as a technology are nicely recorded in a lecture which Hofmann addressed to teachers (Hofmann, 1861). He admitted that the study of organic chemistry was in its infancy, but he foresaw its potential:

The notion that the action of most of our medicines is chemical, is daily growing into a general conviction. We admit that with every change wrought by pharmaceutical agents in the state of our organism, there occurs a corresponding change in its composition, resulting from their reaction on one or more of its constituents.... Associated with chemistry, medicine no longer draws the veil of vitality over processes, the mystery of which may be unlocked by the key of analysis....

> The special zeal with which the field of organic chemistry has been cultivated during the last thirty years, the simple and accurate methods which we now possess for determining the composition of organic products, the amount of analysis actually performed, and, more than all, the still untiring energy of the numerous labourers in the same field of investigation, hold out the promise that the connextion between medicine and chemistry, becoming daily more intimate, will be productive of benefits, the importance of which we can scarcely venture to estimate in the present state of our knowledge.
> *(Hofmann, 1861, pp. 12–13)*

It is interesting to note the importance which Hofmann assigned to "analysis" and what appears to be a change in its definition compared with that in the quotation from Berzelius.

1.6 Pasteur and organic chemistry

Quite apart from the chemical problems which this work posed, it also suffered from the inadequate contemporary understanding of the biology on which the fermentation technology was based. Fermentations were variable, a factor of considerable economic consequence to the brewers, vintners and distillers alike. This was the problem which Bigo (a local distiller) brought to Pasteur in 1856 shortly after the latter's appointment as professor and dean of the new Faculty of Sciences at Lille in France. Bigo manufactured alcohol from sugar beet, but the fermentations would often become acidic and would yield lactic acid rather than ethanol. It must have been a problem such as this which Fortoul, the French minister for public instruction, had in mind when he wrote to Guilleman, the rector at Lille, that

> Pasteur must guard against being carried away by his love for science, and he must not forget that the teaching of the faculties, whilst keeping up with scientific theory, should, in order to produce useful and far-reaching results, appropriate to itself the special applications suitable to the real wants of the surrounding country.
> *(Vallery-Radot, 1901, Chap. 4)*

(Today's politicians would seem to give themselves too much credit for the novelty of their views.)

The work which Pasteur did for Bigo is usually credited with firing his enthusiasm for fermentation. He discovered (Pasteur, 1858b) a new organism (*levûre lactique*) whose cells were much smaller than those of brewer's yeast, and which was always associated with lactic acid fermentation. In another experiment he recovered a micro-organism from aqueous

suspensions of crude calcium tartrate which would ferment tartaric acid [D-tartrate (4)]. The process was easy to follow with a polarimeter as the tartrate was decomposed. However, when he repeated the experiment with racemic acid (DL-tartrate), only the D-tartrate was consumed, and he was able to recover crystalline L-tartrate (5) (Pasteur, 1858a). He noted that this was an excellent method of preparing L-tartrate, and two years later, after repeating the process with *Penicillium glaucum*, he wrote that "it recommends itself as a method, probably of very general application, for splitting apart organic bodies in which it would be reasonable to suppose a molecular composition of the same nature as that of paratartaric acid" (Pasteur, 1860, p. 299). In doing this, Pasteur had put in place the third of his methods for separating optical isomers, following the actual separation of the different crystals (object and mirror-image forms) and the use of an optically active natural base such as quinine to form diastereomeric salts.

L-(+)-(*R*,*R*)- (4) D-(−)-(*S*,*S*)- (5)

tartaric acid

It seems likely that Pasteur's original reason for performing some of these microbiological experiments had more to do with his study of the effects of optically active nutrients on the forms of the organisms which could use them, rather than as an investigation of the fermentation itself (Root-Bernstein, 1989). He wondered whether molecular form could influence the shape of living organisms in the same way as it did the shape of crystals. The outcome of the experiments that followed was rather different, but no less significant. He realized that just as the nature of the yeast, either brewer's or lactic, would divert the fermentation of sugar to alcohol or to lactic acid, so the nature of the nutrient on which the yeast grew could also affect the course of the fermentation. Observations such as these were to lead to his proposition that the course of each fermentation was determined by the organisms which it contained, and that the process itself was dependent on the action of a living organism.

It is necessary to ignore most of what is now taken for granted about fermentation to imagine the powerful effect of Pasteur's work. The advance

in the latter half of the twentieth century which has had an equivalent effect on the science and technology of microbiology must surely be the discovery of the connection between DNA and the genetic code. They brought about similar revolutions in microbiology. Pasteur's concept of the fermentation process itself was in complete contrast to that of Leibig, whose mechanistic attitude to the action of yeast actually delayed advances in brewing practice:

> ... the a priori ideas of Leibig... owing to the genius and commanding authority of their great apostle, had acted as a bar to progress and prevented a dispassionate consideration of the new vitalistic theories of fermentation which were destined to revolutionise all our conceptions of such phenomena.
> *(Brown, 1916, p. 275)*

There is no need here to describe the dispute on the nature of fermentation which subsequently arose between Leibig and Pasteur. There are interesting accounts of the influence of the debate reported by their contemporaries (Sykes, 1895; Frankland, 1897; Brown, 1916). It was finally resolved 20 years later with the publication of *Etudes sur la bière* (Pasteur, 1876), which set out a modern theory of fermentation practice.

The influence of Pasteur's work on organic chemistry is underrated compared with his influence on the study of fermentation and infectious disease. Fermentation became a source of compounds for study, and the separation of isomers was extended to a much larger range of compounds and reactions. Plimpton (1881) prepared a series of amines from chiral and achiral pentanol (amyl alcohol), and Frankland (1885) was able to review a number of chemical changes in their relation to micro-organisms. He noted that there were two types of chemical changes, those effected when two or more substances came into contact, and those effected by contact with a substance which itself was unaltered:

> Failing any satisfactory explanation, very heterogeneous changes of the latter kind have been grouped together under the designation of "catalytic reactions", but a careful study of many of the reactions of this second class has transferred them to the first, and it is more than probable that the remainder, when better known, will be similarly disposed of. The chemical changes occurring in animal and vegetable organisms were, until recently, tacitly, if not formally relegated to the second type. The plant or animal was regarded as effecting the changes by mere contact, or by some mysterious process outside the ken of experimental enquiry. This illusion has been finally dispelled by the synthetical operations of organic chemistry, which have taught us how to produce, by purely laboratory processes, numerous compounds formerly obtainable only as the products of living organisms, and it is to be hoped that chemists and biologists will now give more attention

Table 1.3. *Frankland's list of soluble ferments (1885)*

Soluble ferment	Substrate	Products	Enzyme present in soluble ferment
Diastase	Starch	Dextrin & glucose	Amylase
Invertin	Cane sugar	Glucose & laevulose	Invertase
Synaptase	Salicin	Glucose & saligenin	Glucosidase
Emulsin	Amygdalin	Glucose, benzoic acid & HCN	
	Arbutin	Glucose & hydroquinone	
	Helicin	Glucose & salicylic hydride	
	Phloridzin	Glucose & phloretin	
	Esculin	Glucose & esculetin	
	Daphnin	Glucose & daphnetin	
Pancreatic ferment	Fat	Margaric acid & glycerin	Lipase

to this hitherto neglected field of chemical action – the chemical changes which occur in animal and vegetable organisms.
(Frankland, 1885, p. 159)

Frankland believed that the chemical actions of living organisms were of two kinds, synthetical and analytical (in the sense used by Berzelius), the first being chiefly performed by plants, and the latter by animals, with micro-organisms belonging to the second category. He reviewed a range of processes effected on the one hand by the soluble ferments (Table 1.3), and on the other by the micro-organisms themselves. He noted that all of these processes had their chemical analogies, but that the "organised ferments" were able to carry out processes for which there was no direct chemical equivalent, although there might be some indirect chemical method of performing the same transformation. Most of the reactions which he listed in the latter category were fermentations producing succinate or butyrate from a range of substrates, amongst them Leibig's experiment already described and the synthesis of succinate and glycerol from glucose (Scheme 1.4). By now the atomic composition was correct even if the molecular structure was not appreciated. However, he specifically excluded from this catalogue the power of micro-organisms "to destroy one of the optically active compounds in a mixture, and thus to isolate the compound of opposite optical activity" (Frankland, 1885, p. 181).